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Frog Virus 3 DNA Replication Occurs in Two Stages

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Viral DNA synthesis in frog virus 3 (FV3)-infected cells occurs both in the nucleus and in the cytoplasm (Goorha et al., Virology **84**:32–51, 1978). Relationships between viral DNA molecules synthesized in these two compartments and their role in the virus replication were examined. The data presented here suggest that (i) FV3 DNA replicated in two stages and (ii) nucleus and cytoplasm were the sites of stages 1 and 2 of DNA replication, respectively. Stages 1 and 2 were further distinguished by their temporal appearance during infection and by the sizes of the replicating DNA as determined by sedimentation in neutral sucrose gradients. In stage 1, replicating molecules, between the size of unit and twice the unit length, were produced early in infection (2 h postinfection). In contrast, stage 2 of DNA replication occurred only after 3 h postinfection, and replicating molecules were large concatemers. Results of pulse-chase experiments showed that the concatemeric DNA served as the precursor for the production of mature FV3 DNA. Denaturation of concatemeric DNA with alkali or digestion with S1 nuclease reduced it to less than genome size molecules, indicating the presence of extensive single-stranded regions. Analysis of replicating DNA by equilibrium centrifugation in CsCl gradients after a pulse-chase suggested that these single-stranded regions were subsequently repaired. Based on these and previous data, a scheme of FV3 replication is presented. According to this scheme, FV3 utilizes the nucleus for early transcription and stage 1 of DNA replication. The viral DNA is then transported to the cytoplasm, where it participates in stage 2 DNA replication to form a concatemeric replication complex. The processing of concatemers to produce mature viral DNA and virus assembly also occurs in the cytoplasm. This mode of replication is strikingly different from any other known DNA virus.

Frog virus 3 (FV3) is a large DNA virus of the family Iridoviridae (15). The genome is a linear, double-stranded DNA with a molecular weight of 100×10^6 (11). Originally believed to replicate only in the cytoplasm (12), FV3 has since been shown to require a functional nucleus for its replication (9). Recent work has shown that the FV3 genome enters the nucleus, where it is transcribed during early stages of replication (8) probably by a modified host RNA polymerase II (6). Although the viral DNA replicates in both the nucleus and the cytoplasm (8), virus assembly and morphogenesis take place only in the cytoplasm (7, 12).

A dilemma in this scheme of FV3 replication is posed by the two sites of viral DNA replication. Why should FV3 DNA be synthesized in the cytoplasm and the nucleus? What are the relationships between the DNA molecules synthesized in these cellular compartments, and how do these two sites of DNA replication fit into the overall replication scheme of FV3? The data presented in this paper provide an explanation. FV3 DNA replicated in two stages; each was distinguished by the size and the site of replicating viral DNA. Stage 1 DNA synthesis was restricted to the nucleus, and the size of the replicating DNA ranged from genome size to twice genome size. Stage 2 DNA replication occurred in the cytoplasm exclusively, and the replicating DNA was concatemeric in size. To integrate these data, I have proposed a replication scheme in which the nucleus is a site for early transcription and stage 1 DNA replication; concatemeric DNA, which is presumably involved in virus morphogenesis, is only synthesized in the cytoplasm.

MATERIALS AND METHODS

Cells and virus. Fathead minnow cells were grown as monolayers in 60-mm dishes at 33°C in Eagle minimum essential medium containing 10% fetal calf serum (MEM-10). A clonal isolate of FV3 was grown and assayed at 30°C in fathead minnow cells as described previously (17).

Isotopic labeling of DNA in infected cells. Fathead minnow cell monolayers were infected at a multiplicity of infection of 20 PFU per cell at 23°C for 60 min. After

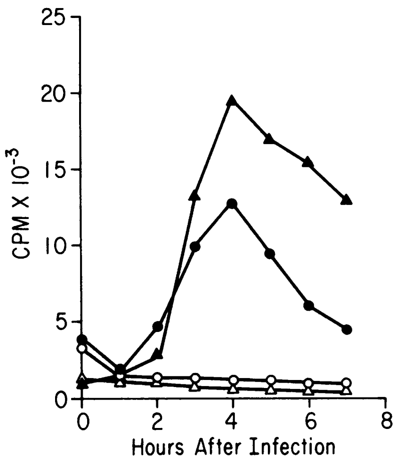

FIG. 1. DNA synthesis in the nucleus and cytoplasm of FV3-infected cells. Fathead minnow cells were infected as described in Materials and Methods. At the indicated times, the cells were labeled with [^{3}H]thymidine (10μCi/ml) for 5 min. Purified nuclear and cytoplasmic fractions were prepared as previously described (8). Trichloroacetic acid-precipitable radioactivity was collected on Millipore filters, which were then washed three times with trichloroacetic acid, dried, and counted. Symbols: (●) nuclear fraction, active FV3; (○) nuclear fraction, heated FV3; (▲) cytoplasmic fraction, active FV3; and (△) cytoplasmic fraction, heated FV3.

adsorption, the cells were washed and overlaid with MEM containing 2% dialyzed fetal calf serum (MEM-2). This time was considered 0 h for the growth cycle. In the initial experiments, cells were first exposed to heat-inactivated FV3 and then infected with active virus to inhibit cellular DNA synthesis as previously described (8). In the current series of experiments, however, cells did not receive heat-inactivated FV3, because host DNA synthesis could be effectively inhibited by the active virus alone (Fig. 1).

At various intervals after infection, the medium was replaced with MEM-2 containing 10 μCi of [^{3}H]thymidine per ml. After the cells were labeled, they were washed three times with phosphate-buffered saline (PBS) and processed for sucrose or CsCl gradient analysis.

Velocity sedimentation of DNA in sucrose density gradients. Cell monolayers were directly lysed in dishes by 0.5% Sarkosyl solutions in hypotonic buffer (0.01 M NaCl–0.005 M EDTA–0.001 M KPO_4 [pH 7.3]). In some cases, DNA extracted from [^{14}C]thymidine-labeled purified FV3 virions was added into the lysed cells. The samples were heated at 60°C for 15 min and incubated at 37°C for 2 h after mixing with an equal volume of pronase solution (2 mg/ml, self-digested for 2 h at 37°C). Crude nuclear and cytoplasmic fractions (see Fig. 7) received Sarkosyl to a final concentration of 0.5% before heat and pronase treatment. A sample was layered on 5 to 20% (wt/vol) linear gradients of either neutral sucrose (in 1.0 M NaCl–0.003 M EDTA–0.05 M Tris [pH 7.4]–0.1% Sarkosyl) or alkaline sucrose (in 0.70 M NaCl–0.3 N NaOH–0.003 M EDTA) and centrifuged in an SW27 rotor as indicated in each experiment. Fractions of 1 ml were collected, and trichloroacetic acid-precipitable radioactivity was determined as previously described (8).

Equilibrium centrifugation in CsCl density gradients. The DNA obtained as described in the previous section was also used to determine buoyant density by centrifugation to equilibrium in CsCl density gradients. The CsCl solution was prepared by adding 6.5 g of CsCl in 5 ml of buffer solution (0.01 M Tris-hydrochloride [pH 7.8]–0.001 M EDTA). A portion of the sample (not more than 50 μl in volume) was mixed with the CsCl, and centrifugation was performed in an SW65 rotor at 115,000 × g for 72 h at 20°C. Fractions (170 ± 25 μl in volume) were collected from the bottom of the tube. The DNA was precipitated on nitrocellulose filters with 10% trichloroacetic acid, and acid-precipitable radioactivity was determined.

Extraction of DNA with phenol-chloroform. DNA from infected cell cultures or purified FV3 preparation was extracted with phenol-chloroform. FV3 virions were purified as previously described (8). The number of nicks in the viral DNA was kept minimal by not freezing, sonicating, or pelleting the FV3 preparation at any time during the purification procedure. DNA was extracted by the procedure of Sarov and Freedman (19) with some modifications. Purified FV3 in low-salt buffer (0.01 M Tris [pH 7.5]–0.01 M EDTA) or lysed cells were treated with proteinase K (self-digested for 2 h at 37°C; 1 mg/ml) and 0.5% sodium dodecyl sulfate for 3 h at 37°C. Sucrose and NaCl were then added to a final concentration of 27% (wt/vol) and 1.0 M, respectively. The solution was extracted twice with phenol. The phenol phase was removed, and the solution was again extracted twice with chloroform. The aqueous layer was carefully removed, and the DNA preparation was dialyzed against low-salt buffer for 48 h with four changes of the buffer.

RESULTS

Kinetics of FV3 DNA synthesis. The kinetics of viral DNA synthesis were determined to provide the background information on the sites and the time course of DNA replication in the infected cells for the subsequent experiments. The rate of [^{3}H]thymidine incorporation into trichloroacetic acid-precipitable radioactivity at indicated times postinfection (p.i.) is shown in Fig. 1. In accord with the previous observations (8), cells exposed to heated FV3 showed severe inhibition of cellular DNA synthesis by 1 h p.i. Additionally, DNA-DNA hybridization results also indicated that more than 90% of the newly synthesized DNA in the infected cells was virus specific (data not shown). Viral DNA synthesis began at about 2 h p.i., reached a peak rate at 4 h p.i., and declined thereafter. At the peak of DNA synthesis, FV3-infected cells incorporated about 40 times more radioactivity into DNA than did cells exposed to heated FV3 (56°C for 20 min). Both the nucleus and the cytoplasm showed similar patterns of DNA synthesis. These results establish that newly synthesized viral DNA is found both in the nucleus and the cytoplasm, in agreement with the earlier studies of FV3 DNA replication (8).

Evidence for two stages in FV3 DNA replication. DNA replication in T (reviewed in reference 16) and lambdoid (reviewed in reference 20) bacteriophages occurs in two stages. In stage 1, progeny DNA is genome length or less, whereas in stage 2, it appears as a large concatemeric form that permits daughter molecules to regenerate their 3′ ends (23) or become circularly permuted, and terminally redundant (21, 20). Since FV3 genome is circularly permuted and terminally redundant (8a), I have looked for concatemer formation and hence two stages of DNA replication. To minimize the generation of artifacts during the experimental procedure, total unfractionated cells were first used to establish the physical characteristics of the replicating DNA.

FV3-infected cells were labeled with [^{3}H]thymidine for 10 min at various times after infection, and sizes of the replicating DNA molecules were determined by sedimentation in neutral sucrose gradients. Figure 2 shows that cellular DNA synthesis is almost completely inhibited by 1 h p.i. under the experimental conditions. Viral DNA synthesis became detectable by 2 h p.i. In early phases of infection (up to 3 h p.i.), the size of the replicating DNA was quite heterogenous. However, most of the newly synthesized DNA sedimented up to twice the size of mature viral DNA. By contrast, at 4 h p.i., most of the newly synthesized DNA was pelleted, suggesting that the replicating DNA was very large in size and probably had a concatemeric form. Since replicating progeny DNA would remain associated with the parent template DNA in neutral sucrose gradients, the above results suggest that progeny DNA was synthesized up to 3 h p.i. in unit length or less than unit length, whereas late in infection (4 h and later), progeny DNA replicated as a concatemeric structure. The amounts of radioactivity incorporated into different size replicative structures at various times after infection are given in Table 1. Up to 3 h p.i., about 65% of the replicating DNA sedimented between 60S and 110S, and about 20% sedimented at S values less than that of mature viral DNA (60S). However, at 4 h or later, the size of the replicating molecules shifted abruptly. Now, only about 15% of the replicating DNA was found in the 60S to 110S range, and about 70% of radioactivity was found in the pellet, indicating that most of the replicating molecules late in infection formed large replicative complexes. It should be pointed out that appearance of newly synthesized viral DNA in large complexes does not result from renaturation during the extraction or trapping, because

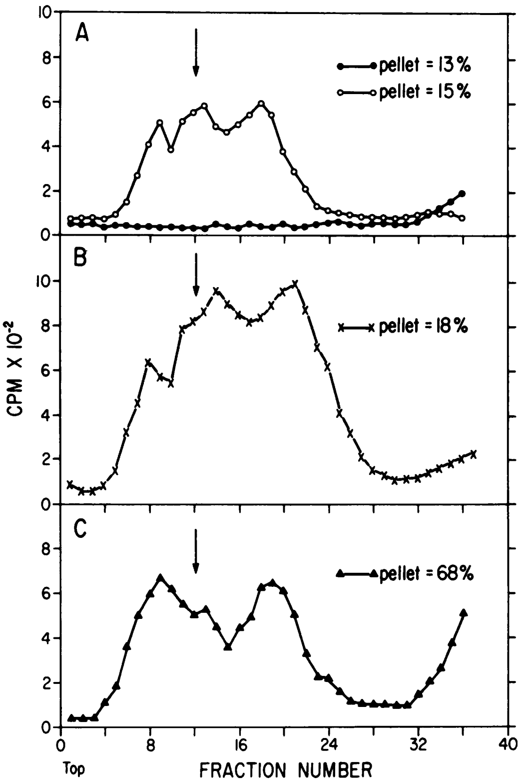

FIG. 2. Sedimentation characteristics of replicating DNA in neutral sucrose gradients at various times after infection. At the indicated times, FV3-infected cells were labeled with [^{3}H]thymidine (10 μCi/ml) for 10 min. After the cells were labeled, they were processed as described in Materials and Methods. Samples were gently layered on 5 to 20% (wt/vol) linear sucrose gradients (1.0 M NaCl–0.003 M EDTA–0.05 M Tris–0.1% Sarkosyl). The gradients were centrifuged for 16 h at 13,200 × g in an SW27 rotor. Fractions of 1 ml were collected, and trichloroacetic acid-precipitable radioactivity was determined. The arrow indicates the position of [^{14}C]thymidine-labeled purified FV3 DNA. (A) infected cells labeled at 1 h (●) or 2 h (○) p.i. (B) infected cells labeled at 3 h p.i. (C) infected cells labeled at 4 h p.i.

(i) it is seen only after 3 h p.i., (ii) [^{14}C]thymidine-labeled DNA, extracted from purified virions and added immediately after the lysis of cells with Sarkosyl, was not found in pellet, indicating that there was no renaturation of DNA molecules during the extraction period, and (iii) glyoxal, added to prevent renaturation (13), did not change the results (data not shown). Similarly, it is unlikely that the replicating DNA in the pellet is a condensed or supercoiled form of mature viral DNA, because examination of large replicative complex, isolated and purified by CsCl density centrifugation, by electron micros-

TABLE 1. Sedimentation characteristics of replicating DNA synthesized at various times after infection[a]

Time after infection (h)	% Total radioactivity in S values		
	<Genome length (<60S)	≥Genome length (60–110S)	Pellet
2	19	65	16
3	18	61	21
4	16	17	67
5	16	15	69

[a] Cells were infected, labeled with [^{3}H]thymidine, processed, and centrifuged in neutral sucrose gradients as described in the legend to Fig. 2. From 2 h p.i., 90% or more of the radioactivity was found in viral DNA as determined by DNA-DNA hybridization.

copy showed a very large, tangled structure—much larger than twice the size of mature viral DNA (unpublished results). Thus, FV3 DNA was indeed synthesized in two stages, each distinguished by its time of appearance during the infection cycle and by the size of the replicating molecules.

Presence of single-stranded regions in large replicative complexes. The nature of the material that binds the replicating viral DNA molecules into large complexes was investigated with various enzymes, nonionic detergents, and alkali. Replicating DNA, identified by labeling with [^{3}H]thymidine for 15 min at 4.5 h p.i., was treated with pronase, RNase, Triton X-100, or S1 nuclease (Table 2). In previous experiments (Fig. 2), the lysed cells were treated with pronase before centrifugation in neutral sucrose gradients. Therefore, as expected, further digestion of the replication complexes with pronase did not affect the size distribution of the replicating molecules. Similarly, treatment of newly synthesized DNA with RNase or Triton X-100 did not cause any reduction in the size of the large replicative complexes. Thus, none of these treatments, which should have removed proteins, RNA, or membrane fragments, affected the size of large replication complexes. However, digestion with S1 nuclease reduced most of the rapidly sedimenting DNA into molecules that were smaller than the mature viral DNA (Table 2, Fig. 3). These results suggest that the concatemeric DNA contained single-stranded DNA regions.

The presence of nicks in the replicating DNA was also investigated by analysis in alkaline sucrose gradients (Fig. 4). Almost all of the replicating DNA, when completely denatured, sedimented at S values lower than the mature viral DNA. These results (Fig. 3 and 4) suggest that the large replicative complex, seen late in infection, did not consist of completely ligated, covalently linked monomers of FV3 genomes but instead contained many nicks and gaps. The extent of single-stranded regions in the replicating DNA was determined from changes in its buoyant density, as measured by centrifugation in CsCl gradients (Fig. 5). In these experiments, infected cells were labeled with [^{3}H]thymidine for 10 min at 4.5 h p.i. Chase was performed by washing the replicate cell cultures with warm PBS three times after the labeling period and reincubating the cells in 5 ml of the medium containing unlabeled thymidine (5 mM). The incorporation of [^{3}H]thymidine into the DNA extracted from infected cells labeled for 10 min and chased for 30 min was 1.4 times higher than that observed immediately after the pulse (data not shown). However, there was no further increase in radioactivity during the 60-min chase, indicating that the chase was effective. Figure 5 shows the buoyant density profiles of the labeled DNA. The replicating DNA (10-min pulse) had a demonstrably higher density than the mature viral DNA, indicating the presence of extensive single-stranded regions in the replicating DNA. It is noteworthy that the DNA from purified virions contains nicks and gaps (7; unpublished data); the higher buoyant density of replicating DNA suggests that it contains more

TABLE 2. Effect of enzymatic and chemical treatment on large replicative complex[a]

Treatment	% Total radioactivity in S values		
	<Genome length (<60S)	≥Genome length (60–110S)	Pellet
None	19	15	66
Pronase (2 mg/ml)[b]	17	16	67
RNase (100 μg/ml)[c]	18	18	64
S1 nuclease (100 U/ml)[d]	54	18	28
Triton X-100 (1%)[e]	17	17	66

[a] Infected cells were labeled with [^{3}H]thymidine for 15 min at 4.5 h p.i. The processing of samples and centrifugation conditions were as described in the legend to Fig. 2.

[b] Samples were treated as described in Materials and Methods except that pronase digestion was continued for 6 h.

[c] DNA from the infected cells was extracted with phenol-chloroform as described in Materials and Methods. A portion of the sample was digested with pancreas RNase (100 μg/ml) for 15 min at 23°C. The samples were then centrifuged in neutral sucrose gradients as described in the legend to Fig. 2.

[d] Conditions for the enzyme digestion and centrifugation in neutral sucrose gradients are described in the legend to Fig. 3.

[e] Samples were processed as described in Materials and Methods, except that after the pronase digestion Triton X-100 was added to the final concentration of 1%.

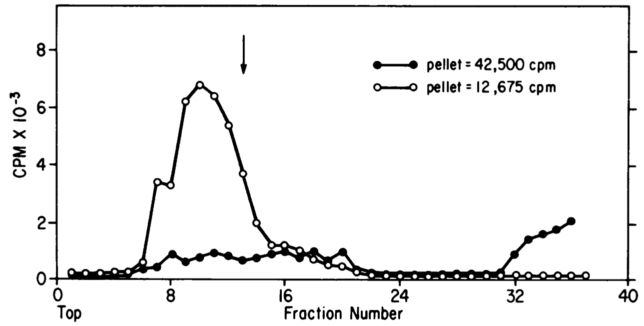

FIG. 3. Sedimentation characteristics of viral DNA in neutral sucrose gradients; released from the large replication complex after S1 nuclease digestion. FV3-infected cells were labeled for 15 min with [^{3}H]thymidine at 4.5 h p.i. After the labeling period, cells were washed three times with PBS, and DNA was extracted with phenol-chloroform (details in Materials and Methods). The DNA preparation was dialyzed against 0.1 M NaCl–0.03 M sodium acetate (pH 4.5)–10 mM $ZnSO_4$ buffer. A sample was mixed with S1 nuclease (final concentration, 100 U/ml), and incubation was performed at room temperature (23°C) for 15 min with gentle shaking. The samples were then heated for 15 min at 60°C and analyzed on neutral sucrose gradients as described in the legend to Fig. 2. Sedimentation analyses were performed within 1 h of S1 nuclease treatment. Symbols: (○) sample was treated with S1 nuclease; (●) control (no S1 nuclease).

extensive single-stranded regions than found in virion DNA. The density of replicating DNA shifted to that of mature viral DNA after a chase period of 60 min, suggesting that during this period most of the gaps and nicks were repaired.

Large replicative complex as precursor of mature viral DNA. In several bacteriophages (16, 20, 22) and animal viruses (2, 5, 10), DNA from the large replicative complex is processed into mature circularly permuted (21, 22) or noncircularly permuted (2, 5, 10, 20) genomes. Therefore, I examined the possibility that the large replicative complex of FV3 acted as a precursor for the production of mature viral DNA. In the following experiment, infected cells were labeled for 15 min at 4.5 h p.i. and chased for 60 min in the presence of 5 mM unlabeled thymidine. The DNA extracted from these cells was analyzed by sedimentation in neutral sucrose gradients. The amount of radioactivity incorporated in the DNA extracted after the chase period was 1.3 times greater than that in the pulse-labeled DNA (data not shown), indicating that the chase was effective, although not complete.

Most of the radioactivity incorporated into the

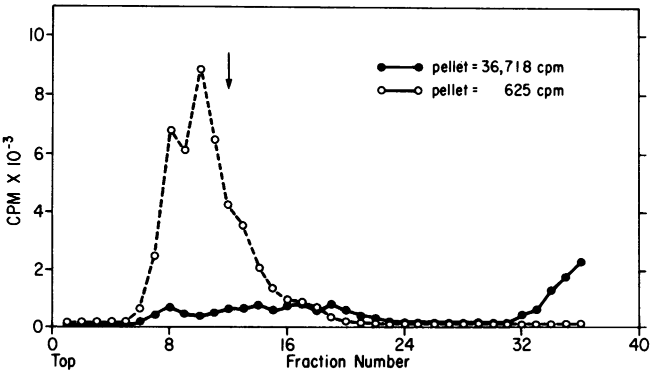

FIG. 4. Sedimentation characteristics of replicating DNA after complete denaturation in alkaline sucrose gradients. FV3-infected cells (labeled for 15 min at 4.5 h p.i.) were processed as described in Materials and Methods. A portion (50 μl) of the processed sample was mixed with 1 ml of 0.5 N NaCl–10 mM EDTA solution and incubated at room temperature (23°C) for 30 min. The DNA-containing solution was then layered on a 5 to 20% (wt/vol) alkaline sucrose gradient (0.70 M NaCl–0.3 N NaOH–0.003 M EDTA) and centrifuged for 16 h at 13,200 × g in an SW27 rotor. For comparison, a portion of the processed sample was also centrifuged in neutral sucrose gradient for 16 h at 13,200 × g in an SW27 rotor. Arrow indicates the position of mature FV3 DNA in neutral sucrose gradient. The other details are given in the legend to Fig. 2. Symbols: (●) Distribution of radioactivity in neutral sucrose gradient; (○) distribution of radioactivity in alkaline sucrose gradient.

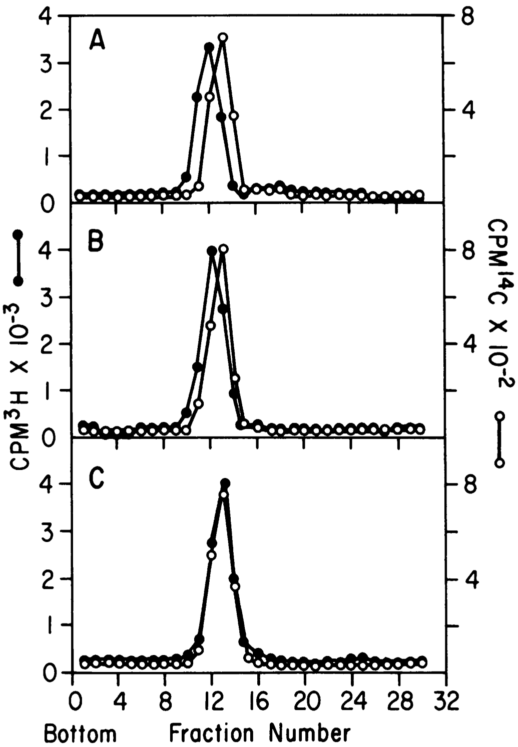

FIG. 5. Buoyant density of the replicating and pulse-chased viral DNA in CsCl equilibrium density gradients. Infected cells were labeled with [^{3}H]thymidine (10 μCi/ml) for 10 min at 4.5 h p.i. Replicate cell cultures were washed three times with PBS and reincubated in chase medium (MEM-2 containing 5 mM thymidine) for 30 or 60 min. Cells were processed as described in Materials and Methods, and samples were centrifuged in an SW65 rotor for 72 h at 115,000 × *g*. The profiles shown are DNAs extracted from the infected cells after a 10-min pulse (A) and then chased for 30 (B) or 60 min (C). [^{14}C]thymidine-labeled virion FV3 DNA was added to each sample as a marker DNA.

DNA during the pulse-labeling period was found in the pellet fraction (Fig. 6). However, during the 60-min chase, about 70% of this large DNA was converted into DNA that sedimented at the position of virion DNA. As seen in previous experiment (Fig. 2), 15 to 20% of the radioactivity was incorporated into DNA smaller than genome length. However, this fraction of radioactivity did not change appreciably after the chase. It is noteworthy that virtually no radioactivity released from the large replicative complex was found in DNA molecules larger than genome size but smaller than the replicative complex. These results argue against a random breakdown of large replicative complexes due to DNase action and support the idea that the DNA in the large complex is processed into mature viral DNA.

Infected cells were also labeled for 15 min at 4.5 h p.i. and chased in a medium containing unlabeled thymidine (5 mM) and cycloheximide (100 μg/ml) to determine whether the maturation of viral DNA from the concatemers to the genome length required protein synthesis. About 75% of the radioactivity was released from the large replicative complex after a chase of 1 h (Fig. 6). However, the size of most of the released DNA was smaller than genome-length molecules. Although this DNA is subgenomic in size, there was no evidence for a random breakdown of the DNA within the large replicative complex. These results indicate that in the absence of protein synthesis, the normal conversion of large replicating DNA into mature viral DNA was disrupted and most of the replicating DNA is cleaved into subgenome-size molecules.

Evidence that stages 1 and 2 of DNA replication are restricted to the nucleus and the cytoplasm, respectively. Since newly synthesized FV3 DNA was found in both the nuclear and cytoplasmic fractions (Fig. 1), I next investigated the sizes of replicating DNA molecules in both of these cellular compartments. Infected cells were labeled with [^{3}H]thymidine for 15 min at 2 or 4.0 h p.i. After the cells were labeled, they were separated into crude nuclear and cytoplasmic fractions, and replicating DNA was analyzed by centrifugation in neutral sucrose gradients (Fig. 7).

Two observations were pertinent to the location of stages 1 and 2 in DNA replication. In the cytoplasmic fraction newly synthesized DNA was found mainly in large replicative complexes at 4 h p.i. only; in the nuclear fraction, a substantial amount (about 80%) of newly synthesized DNA was in small replicating complexes at 2 h p.i. and at 4 h p.i. Any interpretation of these observations must be accompanied by two caveats. First, almost 20% of the radioactivity of the nuclear fraction was found in the pellet. This fraction of newly synthesized DNA most probably represented residual cellular DNA synthesis and contamination of nuclear fraction by the cytoplasmic material. Second, about 20% of the radioactivity of the cytoplasmic fraction was distributed over a broad size range of DNA molecules: less than genomic, genomic, and more than genomic (but smaller than large replication complex). This fraction of newly synthesized DNA most probably represents sum of three activities: (i) breakdown of concatemers during the fractionation procedure; (ii) conversion of stage 1 progeny DNA to large replicative complex after transport to the cytoplasm; and (iii) processing of concatemers into mature viral DNA during the labeling period.

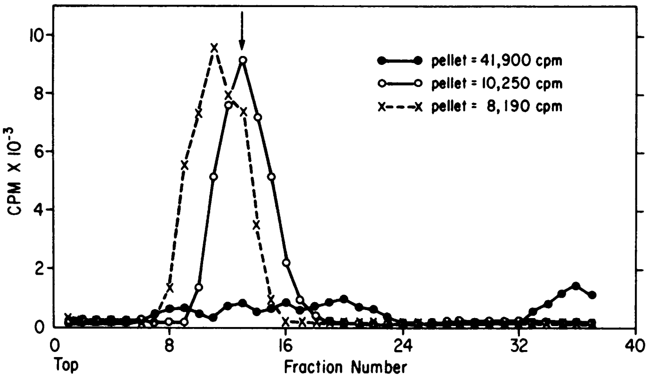

FIG. 6. Sedimentation characteristics of viral DNA released from large replicative complexes in neutral sucrose gradients. Infected cells were labeled for 15 min at 4.5 h with [^{3}H]thymidine (10 μCi/ml). Replicate cell cultures were washed three times with PBS and reincubated in chase medium (MEM-2 containing 5 mM thymidine) for 60 min. The other details are given in the legend to Fig. 2. Symbols: (●) 0-h chase; (○) 60-min chase; and (×), 60-min chase in presence of cycloheximide (100 μg/ml).

It should be emphasized here that crude nuclear and cytoplasmic fractions, obtained by low-speed centrifugation of cells disrupted in a Dounce homogenizer, were used in the experiment shown in Fig. 7. Thus, most probably, there was cross-contamination of one fraction by another. Attempts to purify crude nuclei by treatment with Triton X-100, Nonidet P-40, or centrifugation of crude nuclei through 0.3 or 1.0 M sucrose solutions were unsuccessful, resulting in the breakdown of replicating molecules (data not shown). Even in crude nuclear and cytoplasmic preparations, there was some breakdown of replicating DNA, particularly of large replication complex. Varying the temperature or Mg^{2+} in the fractionation procedure did not prevent breakdown. Fragility of replicating DNA has also been reported in herpesviruses (2).

Despite these qualifications, the conclusion most compatible with the data in Fig. 7 is that during stages 1 and 2 of replication, viral DNA was indeed synthesized at separate sites. Thus, stage 1 of DNA replication, characterized by a

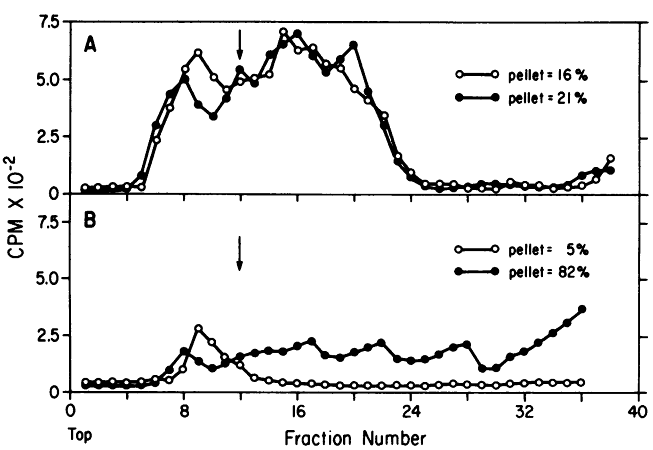

FIG. 7. Sedimentation characteristics of replicating DNA in neutral sucrose gradients obtained from the nuclear and cytoplasmic fractions of infected cells. Infected cells were labeled with [^{3}H]thymidine for 15 min at 2 or 4 h p.i. After the labeling period, cells were washed three times with PBS and were scraped into 1 ml of hypotonic buffer (0.01 M NaCl–0.005 M EDTA–0.001 M KPO_4 [pH 7.3]) with a rubber policeman. The cells were allowed to swell for 10 min at 4°C and were then disrupted by a tight-fitting Dounce homogenizer (40 strokes). The disrupted cells were centrifuged at 1,000 × g for 2 min. The resuspended pellet constituted the crude nuclear fraction, whereas supernatant fluid was considered as the cytoplasmic fraction. The other details are given in the legend to Fig. 2. Nuclear fraction (A); cytoplasmic fraction (B). Symbols: (○), cells labeled at 2 h p.i.; (●) cells labeled at 4 h p.i.

small replicating complex, was restricted to the nucleus; whereas stage 2, characterized by a large replicating complex, occurred exclusively in the cytoplasm. Additionally, stage 1 DNA replication in the nucleus continued late into infection (4.0 h p.i.). Apparently, the onset of stage 2 DNA replication in the cytoplasm had no effect on nuclear DNA synthesis.

DISCUSSION

There is definitive genetic and biochemical evidence to suggest that in lambdoid (reviewed in reference 20) and T (reviewed in reference 16) bacteriophages DNA replication occurs in two stages. The production of concatemers during stage 2 is required for regeneration of ends in DNA molecules (23) and for packaging them into virions (21, 22). Several viral genes control the transition between the two stages (16, 20). Herpesviruses may also have two stages of DNA replication (2) but unlike lambdoid and T phages, there is no genetic evidence that the transition from stage 1 to stage 2 is controlled by viral genes or that the two stages are obligatory for virion production in animal viruses. The data presented in this paper are consistent with the interpretation that FV3 DNA replication occurred in two stages. During stage 1, progeny DNA was synthesized in unit length or less than unit length, whereas in stage 2, progeny DNA was synthesized as a large concatemeric structure (Fig. 2). The concatemeric structure contained extensive single-stranded regions (Fig. 3, 4, and 5) that were presumably filled before the processing of the concatemer to form mature DNA (Fig. 5). Preliminary characterization of a temperature-sensitive mutant (*ts*12488) of FV3 indicates that this mutant is defective in stage 2 DNA replication (unpublished data). It may, therefore, provide the necessary genetic evidence to confirm a two-stage DNA replication scheme for FV3.

A novel feature of DNA replication in FV3 is that stage 1 is restricted to the nucleus and stage 2 is restricted to the cytoplasm (Fig. 7). It has been previously reported that FV3 DNA is synthesized in both the nucleus and the cytoplasm and that nuclear DNA is transported into the cytoplasm (8). Presumably, then, progeny DNA synthesized in the nucleus during stage 1 is transported to the cytoplasm where it participates in stage 2 DNA replication. The most likely explanation for separate sites of DNA replication in FV3 is that the switch from stage 1 to stage 2 replication requires viral protein(s) that is present only in the cytoplasm. In fact, the cytoplasm of FV3-infected cells contains at least two viral proteins that are not found in the nucleus (4, 14).

Similar to DNA replication in herpesviruses (2, 10) and vaccinia virus (5), FV3 progeny DNA in the large replicative complex served as a precursor for the production of mature viral DNA molecules (Fig. 6). Furthermore, protein synthesis was necessary for the processing of concatemeric DNA. In bacteriophages, processing of concatemeric DNA is associated with the assembly of phage particles (reviewed in reference 1). The most thoroughly studied example of such a relationship is probably phage T4, in which concatemeric DNA is cleaved and then packaged into the virions via a "headful" mechanism. Evidence also suggests that as a consequence of this process, the DNA packaged into the phage heads becomes circularly permuted and terminally redundant (21). A comparable relationship between concatemer processing and packaging or virus assembly, or both, has not been established in animal viruses. However, since the FV3 genome is also circularly permuted and terminally redundant (8a), it is reasonable to assume that this virus may also utilize a similar mechanism. However, experimental evidence for above or any other mechanism remains to be established.

The two-stage sequence of FV3 DNA replication and processing of concatemers can be integrated with previous findings (6, 8, 9) to provide a scheme of replication that contrasts sharply with the replication strategies of most animal viruses (Fig. 8). The genome of incoming FV3 particles reaches the nucleus (7) where it is transcribed during the early stages of infection (8). Cellular RNA polymerase II, modified by structural protein(s) of the virus particles, is probably utilized for viral transcription at this stage (6). The parent genome in the nucleus also serves as the template for stage 1 of DNA replication. Progeny DNA synthesized in the nucleus (or parent genome) is then utilized as a template for further transcription or is transported, or both, to the cytoplasm (8) where it participates in stage 2 of DNA replication. The large replicative complex produced in stage 2 is then cleaved to produce mature viral DNA in the cytoplasm. Presumably, concatemer processing is intimately associated with DNA packaging and virus assembly. This scheme of FV3 replication provides a conceptual framework in which available data on FV3 replication can be accommodated. Several aspects of the replication scheme are not clear at this time. For example, the structure of the replicating molecule (linear versus circular), the size of the replicating molecules transported to the cytoplasm, the mechanisms of concatemer formation, and the site(s) of late transcription are not known. More extensive experimental work will be needed to establish the details of the scheme.

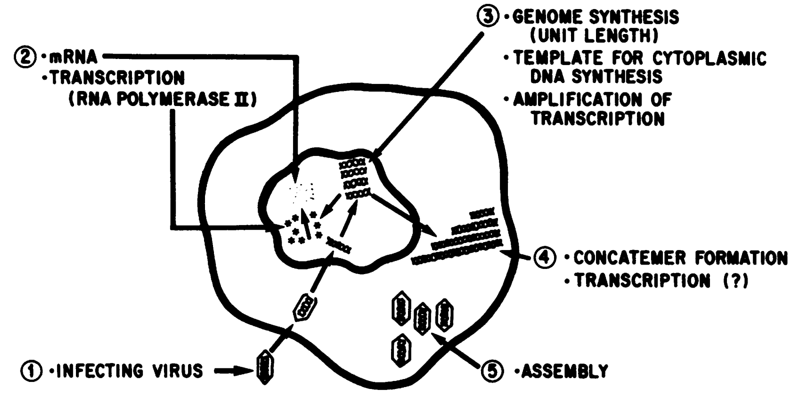

FIG. 8. Proposed replication cycle of FV3. The genomes of parent FV3 particles reach the nucleus (7) where they are transcribed during the early stages of infection (8). Cellular RNA polymerase II, modified by the structural protein(s) of the virus particles, is probably utilized for viral transcription at this stage (6). The parent genomes in the nucleus also serve as the template for stage 1 of DNA replication. Replicative molecules are then transported to the cytoplasm (8) where they participate in stage 2 of DNA replication. The large replicative complex produced in stage 2 is cleaved to produce mature viral DNA. Virus morphogenesis and assembly also occur in the cytoplasm (8, 12).

Two further points, although not directly related to the above conclusions, deserve consideration. (i) The presence of cycloheximide in a pulse-chase experiment (Fig. 6) or treatment of the large replicative complex with alkali (Fig. 4) or S1 nuclease (Fig. 3) generated predominantly subgenomic size fragments. However, the size distribution of the fragmented DNA suggested that the breakdown of replicative complex under these conditions was not random. These results then suggest that the single-stranded regions in the replicative complex were not randomly distributed. The nonrandom distribution of single-stranded regions on phage T4 replicative complex has been accommodated into a "lamp brush" model of DNA replication (3). (ii) Analysis of replicating FV3 DNA by neutral sucrose gradient analysis revealed that a substantial fraction (about 15%) of replicating DNA sedimented as subgenome-size molecules (Fig. 2 and 7, Tables 1 and 2). An obvious possibility is that this fraction represents the breakdown of larger replicating DNA molecules. The homogeneous size of the subgenomic DNA can be explained by assuming that replicating DNA is susceptible to breakdown at sites of replication forks or single-stranded regions, or both, which are not randomly distributed (discussed above). This explanation is also supported by the lack of conversion of this DNA into mature viral DNA or large replicative complexes during the chase (Fig. 6). Alternatively, FV3 DNA might replicate in fragments as suggested for phage T4 (18).

Eukaryotic DNA viruses are classified as cytoplasmic or nuclear depending on whether the nucleus or the cytoplasm is the site for their nucleic acid synthesis and virus assembly. If correct, the replication strategy of FV3 as depicted in Fig. 8 would represent a striking departure from strategies used by other DNA viruses. Phylogenetically, FV3, because it utilizes both the nucleus and the cytoplasm for its replication, may be considered as an intermediate form in the evolution of nuclear and cytoplasmic DNA viruses.

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